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HPLC and GC/MS determination of 4-aminobiphenyl haemoglobin adducts in fetuses exposed to the tobacco smoke carcinogen in utero

Maria T. Pinorini-Godly, Steven R. Myers*

Department of Pharmacology and Toxicology, University of Louisville School of Medicine, Louisville, KY 40292, USA

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Abstract

Maternal-fetal exchange of the potent tobacco-related human carcinogen, 4-aminobiphenyl, was studied in women nonsmokers and in women smokers as well as in the corresponding fetuses during pregnancy. Smoking status of the women in the study was assessed via questionnaire and measurement by immunoassay of serum cotinine in maternal and fetal blood samples. 4-Aminobiphenyl was extracted from both maternal and fetal blood samples using organic solvent extractions and the released amine was qualitatively and quantitatively characterized by analysis of the samples by high pressure liquid chromatography (HPLC) and gas chromatography coupled with mass spectrometry (GC/MS). Background levels (pg 4-aminobiphenyl/g haemoglobin) of 4-aminobiphenyl-haemoglobin adducts were detected in maternal nonsmokers (mean ± S.D; 29.6 ± 16.2 (GC/MS); 23.7 ± 13.5 (HPLC)) and in fetal samples (14.0 ± 6.5 (GC/MS); 10.0 ± 4.6 (HPLC)). Elevated levels of 4-aminobiphenyl-haemoglobin adducts were found in maternal smokers (488 ± 174 (GC/MS); 423 ± 154 (HPLC)) as well as in the corresponding fetal blood samples (244 ± 91 (GC/MS); 197 ± 77 (HPLC)). This study confirms that a potent tobacco-related carcinogen, 4-aminobiphenyl, crosses the human placenta and binds to fetal haemoglobin in significantly higher concentrations in smokers when compared to nonsmokers.

Keywords: 4-Aminobiphenyl; Hemoglobin adducts; Tobacco smoke; Maternal blood; Fetal blood

1. Introduction

Maternal smoking has been shown to be associated with DNA damage in the placenta (Everson et al., 1988) and exposure to tobacco smoke in utero may result in an increase risk of development of childhood and adult cancers (Neutal, et al. 1971; Sandler et al., 1985; Stjernfeldt et al., 1986). A number of aromatic amines, including 4-aminobiphenyl, have been detected in tobacco smoke. Since some of these amines are potent human bladder carcinogens, such as 4-aminobiphenyl and 2-naphthylamine, it is a reasonable hypothesis that increased exposure to these amines is a factor in the observed increase in the incidence of bladder cancer among cigarette

* Corresponding author.

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smokers. The carcinogenicity of 4-aminobiphenyl is believed to derive from the hepatic *N*-oxidation of the parent amine to *N*-hydroxy-4-aminobiphenyl, with subsequent hydrolysis to yield an electrophilic nitrenium ion that binds to either DNA or haemoglobin. In the blood, free *N*-hydroxy-4-aminobiphenyl is oxidized further within the erythrocyte to 4-nitrosobiphenyl, which forms a covalent adduct with haemoglobin (Kadlubar et al., 1988).

Several studies have demonstrated a proportionality between the formation of carcinogen-haemoglobin adducts and the formation of carcinogen-DNA-adducts (Neumann, 1980; Ehrenberg et al., 1983; Shugart and Kao, 1985). Therefore, haemoglobin adduct formation has been proposed as a dosimeter for DNA damage in adult smokers (Bryant and Osterman-Golkar, 1991).

In this study, we investigated the relationship between maternal smoking and 4-aminobiphenyl haemoglobin adduct levels in both maternal and fetal blood. We also present a simple and rapid method for the detection of 4-aminobiphenyl-haemoglobin adducts in paired maternal and fetal blood samples in which the total time for extraction and analysis of the haemoglobin adduct is reduced.

2. Materials and methods

2.1. Chemicals and reagents

4-Aminobiphenyl, and 4′-*F*-aminobiphenyl were purchased respectively from Fluka Chemika-Biochemika (Ronkonkoma, New York) and Sigma-Aldrich Chemical Co. (Milwaukee, WI). All aqueous solutions were prepared with distilled deionized water. Trimethylamine in hexane was prepared by adding 1 g trimethylamine hydrochloride (Fluka Chem.-Biochem.) to 2 ml water, neutralizing the solution with NaOH and extracting with 10 ml hexane. The internal standard, 4′-*F*-aminobiphenyl was recrystallized from dichloromethane/hexane and used to prepare a stock solution of 25 ng/ml in 0.1N HCl which is stored at 4°C. Pentafluoropropionic anhydride (PFPA) was purchased from Fluka. All chemicals and reagents were of the highest grade commercially available.

Blood samples were obtained from Norton's Hospital and the University of Louisville Hospital. Women participating in the study were assessed as to their smoking habits via questionnaire and by assessment by immunoassay (Abbott Laboratories, Abbott Park, IL) of serum cotinine levels. Maternal blood samples (10 ml) were collected using heparinized vacutainers from smoking and nonsmoking mothers during admission for labour and delivery. Fetal blood samples (10 ml) were collected using heparinized vacutainers from the umbilical vein immediately after delivery. Individuals were classified as to their smoking status and were divided into either nonsmokers ($n = 21$) or smokers ($n = 21$). Paired maternal and fetal blood samples were obtained from all individuals in the study.

2.2. Analysis of the samples

Maternal and fetal blood samples were centrifuged at 3000 × *g* to generate packed red blood cells. After removal of serum, the red cells were washed 3 times with 0.9% saline and lyzed by the addition of 15 ml ice cold deionized water and 2 ml toluene with vigorous shaking. After 15 min, samples were centrifuged at 10 000 × *g* for 20 min to remove cellular debris. The haemoglobin solution was transferred to dialysis tubing and dialyzed for 24 h at 4°C against two changes of distilled, deionized water (2 l). Hemoglobin concentrations were determined by measurement of the absorbance at 415 nm (oxyhaemoglobin, $\epsilon = 125$ mM^{-1}). Samples were divided into aliquots (3–5 ml each) to allow for reproducibility of analysis and stored at −20°C until analysis by HPLC and GC/MS.

2.3. Extraction of 4-aminobiphenyl haemoglobin adducts in maternal and fetal blood

Extraction of 4-aminobiphenyl haemoglobin adducts from blood samples followed the method of Del Santo et al. (1991) with modifications. Prior to extraction of the haemoglobin samples for gas chromatographic/mass spectrometric analysis of 4-aminobiphenyl, 400 pg of the internal standard 4′-*F*-aminobiphenyl was added to each sample as an

internal standard. After addition of the internal standard, the haemoglobin solution was made 0.1 M in NaOH and incubated for 2 h at room temperature. The hydrolysate was extracted twice with 15 mls of methylene chloride and the resulting emulsion broken by freezing and thawing the sample. The extracts were treated with 10 μl trimethylamine in hexane and derivatized by the addition of 5 μl pentafluoropropionic anhydride (PFPA) and the resulting derivatized products evaporated under nitrogen. The residue was dissolved in 20 μl hexane and 3 μl injected into the GC/MS for analysis. For the HPLC detection the extraction of the amine was accomplished identically as for the GC/MS method, except that the internal standard was omitted and the extracts were not derivatized prior to analysis.

2.4. Gas chromatographic and mass spectrometric analysis

Gas chromatographic and mass spectral (GC/MS) analysis of the haemoglobin samples was carried out on a Hewlett-Packard 5890 Series II gas chromatograph (GC) connected to a 5971A mass selective detector. The GC oven was fitted with a DB-Wax 20 m capillary column (0.18 mm I.D., 0.3 μm film thickness) operating under the following parameters: 100°C initial temperature for 1 min, ramp rate 20°C/min up to 240°C, held for 15 min (total analysis time = 23 min), injector 200°C, detector (MS) 280°C; inlet pressure of the carrier gas (helium) 3.0 psi. Single ion monitoring was accomplished by detecting the 4-aminobiphenyl-PFP (*m/z* 315) and 4′-*F*-aminobiphenyl-PFP (*m/z* = 333) derivatives. Data analysis was performed on a Hewlett-Packard Vectra QS/20 computer using the HP Chemstation software, version G1034C. Integrated peak areas of 4-aminobiphenyl and derivatives were used to calculate concentrations of 4-aminobiphenyl in the haemoglobin samples.

2.5. High pressure liquid chromatographic analysis

Analysis of 4-aminobiphenyl haemoglobin adducts was accomplished using a Waters M600E solvent delivery system connected to a M996 photodiode array detector and a M470 scanning fluorescence detector. Samples were introduced through a M712 autosampler. The system is controlled through a NEC/33i DX2 computer running the Waters Millennium chromatography manager, version 2.0. A C_{18}-μBondapak column was eluted with a solvent flow rate of 1.5 ml/min using an acidic KCl solution as the weak solvent (10 mM KCl, pH 2.5) and methanol as the strong solvent, with a linear gradient of 20% methanol to 100% methanol in 30 min. UV absorbance was monitored at 254 nm. Quantitation of 4-aminobiphenyl was accomplished by measurement of peak heights and areas corresponding to 4-aminobiphenyl and comparison to a standard curve for 4-aminobiphenyl.

For quantitation of the 4-aminobiphenyl haemoglobin adduct, the GC/MS calibration curve (based on the response factor from 1–600 pg standards prepared and analyzed concurrently with the samples) and the HPLC calibration curve (calculated on the basis of the analysis of a series of external standards) was found to be linear with $r^2 = 0.994$ for the GC/MS calibration curve and $r^2 = 0.915$ for the HPLC calibration curve.

3. Results

The levels of 4-aminobiphenyl adducts in paired maternal and fetal blood samples of smokers ($n = 21$) and nonsmokers ($n = 21$) assessed by the GC/MS and HPLC are shown in Table 1. The concentration of 4-aminobiphenyl detected by GC/MS in maternal blood samples was found to

Table 1
Levels of 4-aminobiphenyl haemoglobin adducts (pg 4-aminobiphenyl/g haemoglobin (Hb)) in maternal and fetal blood samples measured by GC/MS and HPLC

	Mean ± S.D.	
	HPLC (pg 4-ABP/g Hb)	GC/MS (pg 4-ABP/g Hb)
Maternal blood — nonsmokers ($n = 21$)	23.7 ± 13.5	29.6 ± 16.2
Fetal blood — nonsmokers ($n = 21$)	10.0 ± 4.6	14.0 ± 6.5
Maternal blood — smokers ($n = 21$)	423 ± 154	488 ± 174
Fetal blood — smokers ($n = 21$)	197 ± 77	244 ± 91

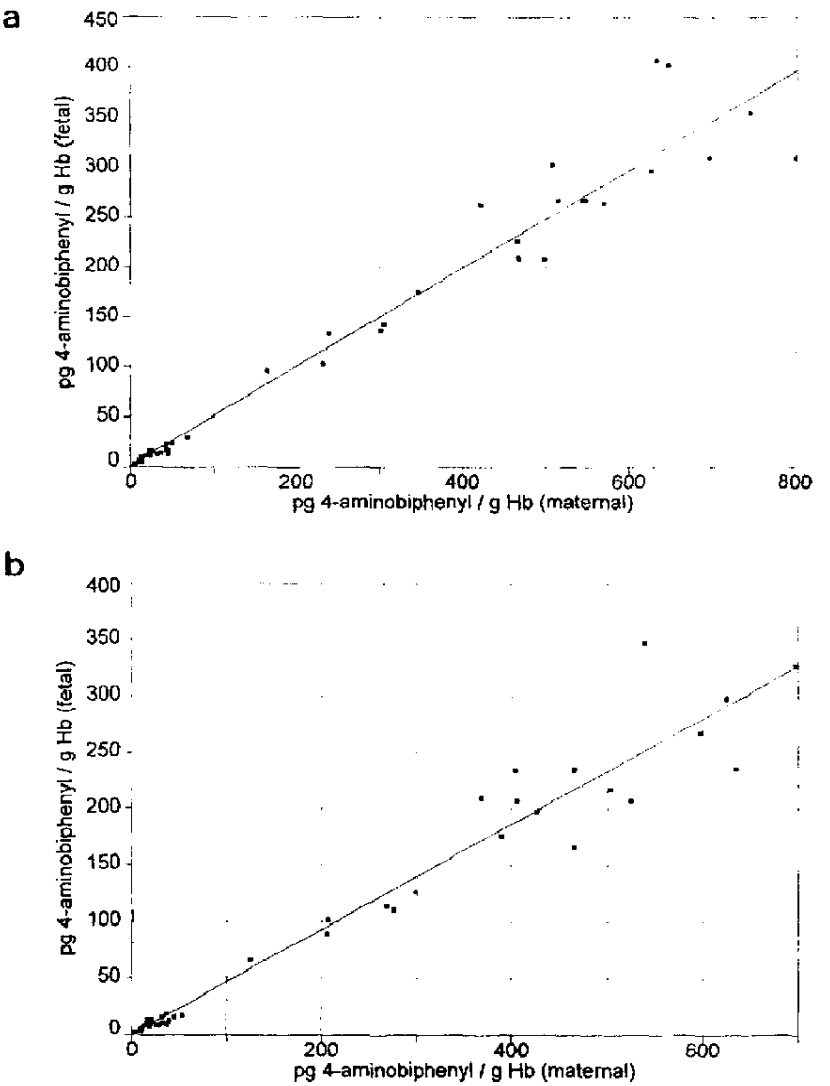

Fig. 1. (a) Linear regression of maternal 4-aminobiphenyl haemoglobin adducts and fetal 4-aminobiphenyl haemoglobin adducts (paired samples measured by GC/MS) in total study ($n = 42$). (b) Linear regression of maternal 4-aminobiphenyl haemoglobin adducts and fetal 4-aminobiphenyl haemoglobin adducts (paired samples measured by HPLC) in total study ($n = 42$).

be significantly higher in smokers (488 ± 174 pg 4-aminobiphenyl/g haemoglobin; mean ± S.D.) compared to nonsmokers (29.6 ± 16.2 pg 4-aminobiphenyl/g haemoglobin). Similarly, the levels of 4-aminobiphenyl haemoglobin adducts detected by GC/MS in fetal blood samples was also significantly higher in smokers (244 ± 91 pg 4-aminobiphenyl/g haemoglobin) when compared to nonsmokers (14.0 ± 6.5 pg 4-aminopbiphenyl/g haemoglobin). In comparison, detection of 4-aminobiphenyl haemoglobin adducts by HPLC resulted in a detection of 423 ± 154 pg 4-aminobiphenyl/g haemoglobin (maternal smokers) and 197 ± 77 pg 4-aminobiphenyl/g haemoglobin

in the corresponding fetal samples. When assayed by HPLC, nonsmokers were found to have adduct levels of 23.7 ± 13.5 (maternal) and 10.0 ± 4.60 (fetal) pg 4-aminobiphenyl/g haemoglobin. The ratio of maternal adduct to fetal adduct in smokers was found to be 2.0 ± 0.3 (GC/MS) and 2.2 ± 0.3 (HPLC) compared to a nonsmokers adduct ratio of 2.1 ± 0.6 (GC/MS) and 2.4 ± 0.8 (HPLC). This adduct ratio is consistent with the ratios reported by Coghlin et al. (1991).

In order to characterize the overall relationship between maternal exposure to the carcinogen 4-aminobiphenyl and fetal exposure, we carried out linear regression on the data from our population using maternal 4-aminobiphenyl haemoglobin adduct as the independent variable and fetal adduct

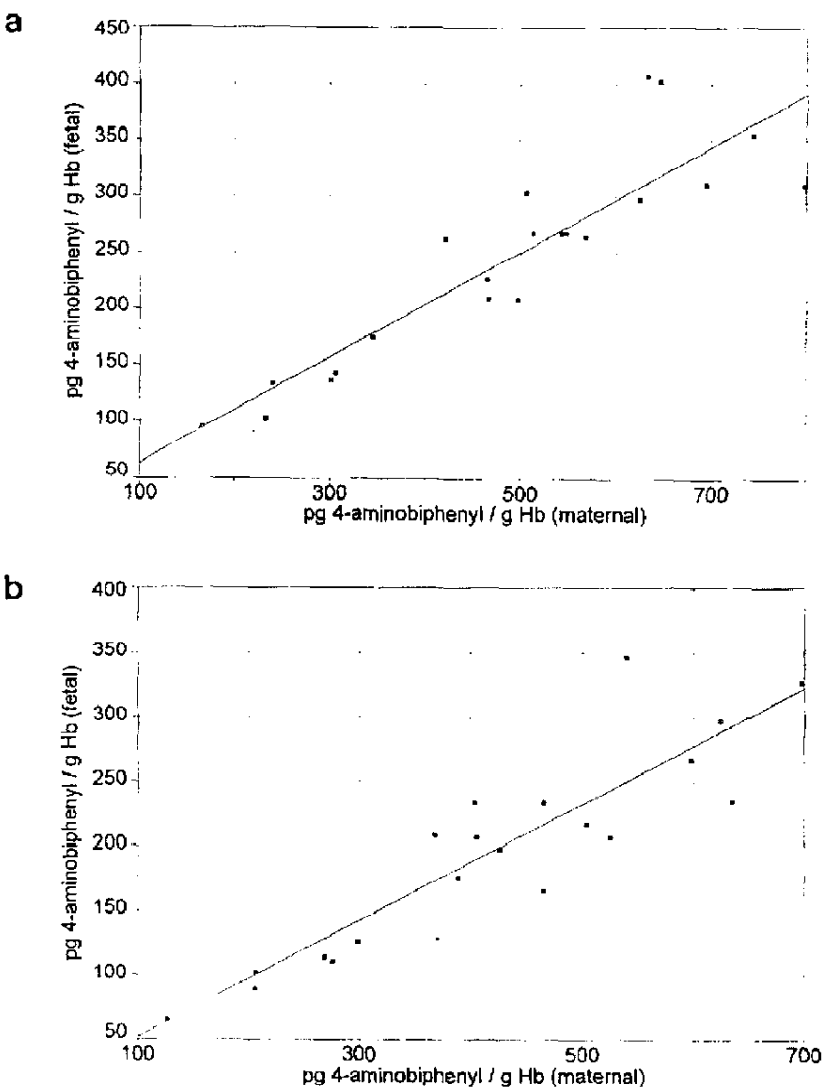

Fig. 2. (a) Linear regression of maternal 4-aminobiphenyl haemoglobin adducts and fetal 4-aminobiphenyl haemoglobin adducts (paired samples measured by GC/MS) in smokers ($n = 21$). (b) Linear regression of maternal 4-aminobiphenyl haemoglobin adducts and fetal 4-aminobiphenyl haemoglobin adducts (paired samples measured by HPLC) in smokers ($n = 21$).

as the dependent variable. When all samples were pooled for analysis (Fig. 1), a significant correlation between maternal and fetal exposures to 4-aminobiphenyl was detected (GC/MS, $r^2 = 0.95$ (Fig. 1a); HPLC, $r^2 = 0.95$ (Fig. 1b)). Separate regression analysis were carried out with each group of paired samples measured by the two methods (HPLC and GC/MS). Detection of maternal and fetal 4-aminobiphenyl haemoglobin adducts in smokers by GC/MS (Fig. 2a) yielded correlation of $r^2 = 0.80$ whereas detection of the adduct by HPLC (Fig. 2b) yielded a correlation of $r^2 = 0.81$.

A typical HPLC chromatogram of 4-

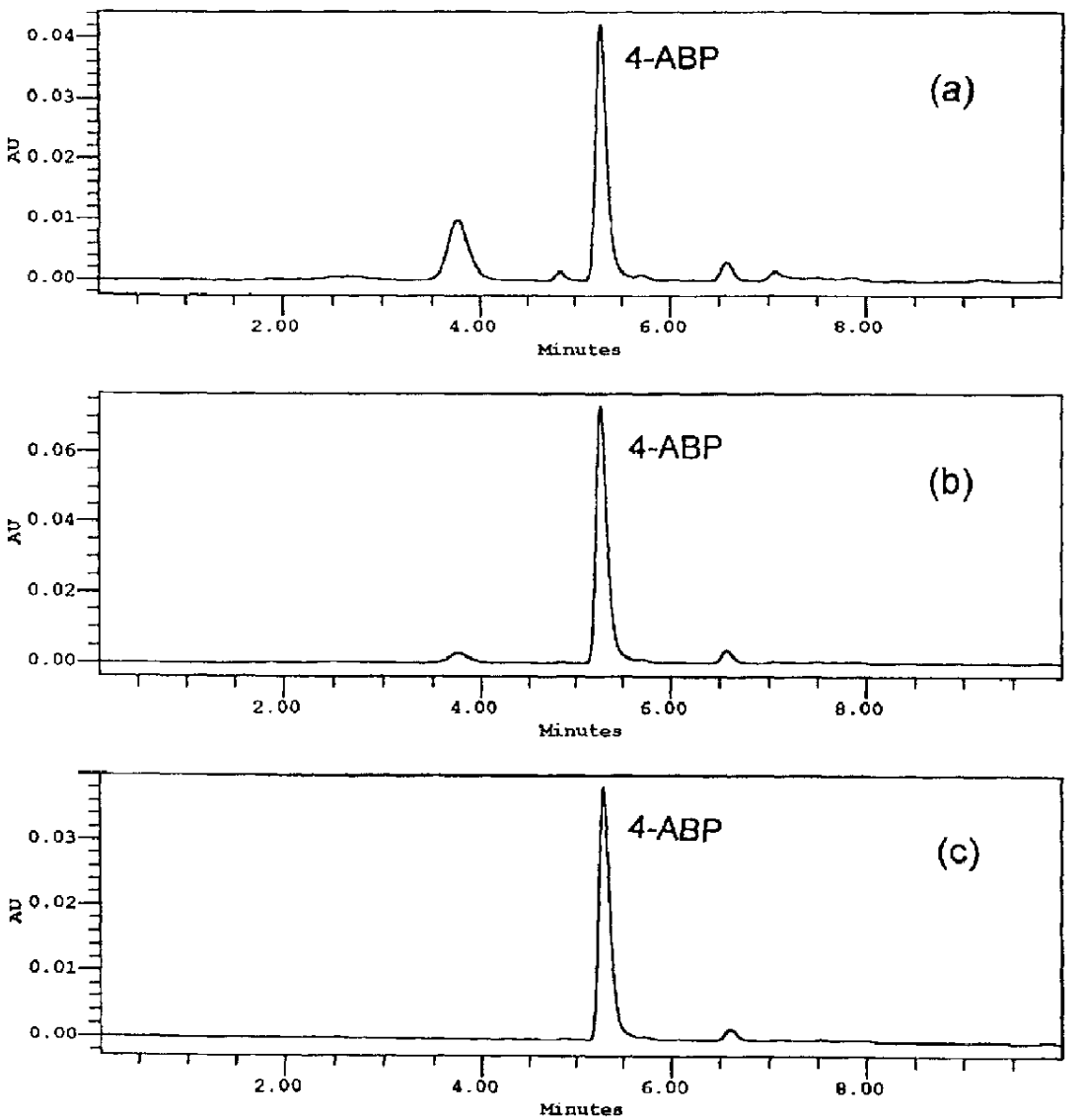

Fig. 3. HPLC spectrum of 4-aminobiphenyl (4-ABP) isolated from maternal blood (a) which is indistinguishable from 4-aminobiphenyl isolated from fetal blood (b) and from the standard 4-aminobiphenyl (c).

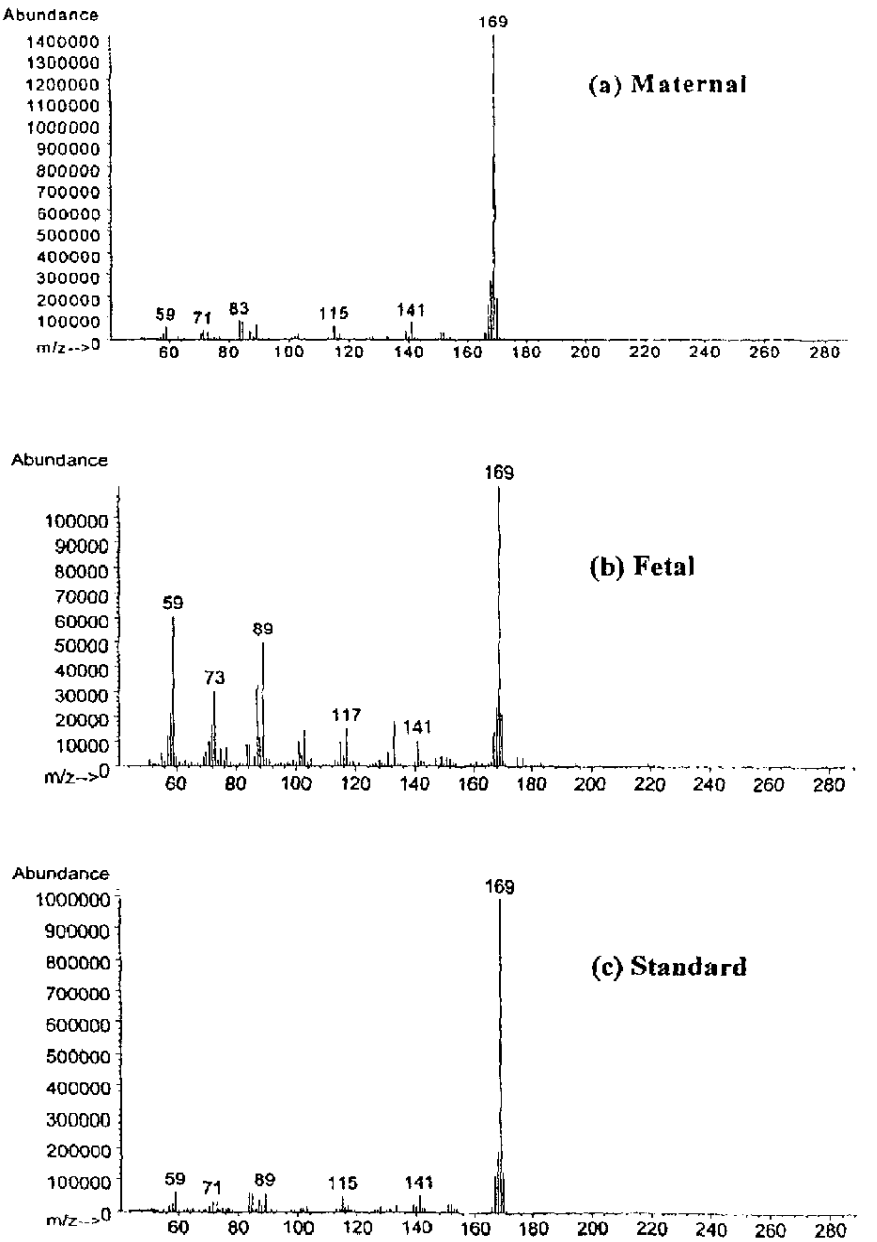

Fig. 4. Full GC/MS spectrum of underivatized 4-aminobiphenyl ($m/z = 169$) isolated from maternal blood (a) which is indistinguishable from 4-aminobiphenyl isolated from fetal blood (b) and authentic 4-aminobiphenyl (c).

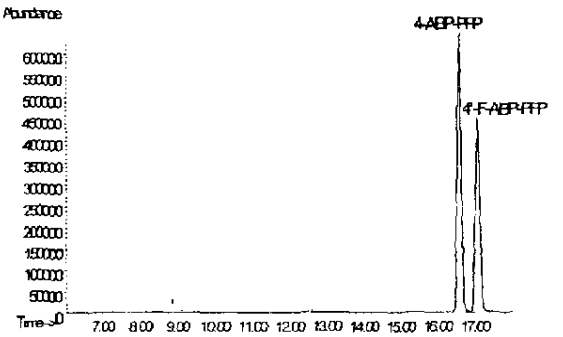

Fig. 5. Typical gas chromatographic profile of 4-aminobiphenyl isolated from blood of smokers. The two peaks at 16.62 min (4-ABP-PFP) and 17.10 min (4′-F-ABP-PFP) correspond to the PFP derivatives of both 4-aminobiphenyl and the internal standard (4′-F-ABP).

aminobiphenyl isolated from maternal smokers blood and the corresponding 4-aminobiphenyl detected from fetal haemoglobin samples is shown in Fig. 3. Both maternal and fetal blood samples yielded a product that co-chromatographed with authentic 4-aminobiphenyl. The fractions identified by HPLC as 4-aminobiphenyl were analyzed by GC/MS (Fig. 4). The mass spectrum yielded a parent molecular ion of *m/z* 169 and accompanying fragmentary ions which were found to be indistinguishable from authentic 4-aminobiphenyl. A typical GC/MS analysis of the extracted 4-aminobiphenyl and the internal reference compound 4′-*F*-4-aminobiphenyl is shown in Fig. 5. The derivatized amine yielded a parent molecular ion of *m/z* of 315 which was consistent with authentic derivatized 4-aminobiphenyl. These results demonstrate that both HPLC and GC/MS detection methods are applicable for the detection of aromatic amine haemoglobin adducts, particularly with 4-aminobiphenyl.

4. Discussion

This study demonstrates that the potent tobacco-related carcinogen, 4-aminobiphenyl, or its active metabolite, *N*-hydroxy-4-aminobiphenyl, crosses the human placenta and binds to fetal haemoglobin. All fetal blood samples tested revealed detectable amounts of 4-aminobiphenyl haemoglobin adducts. Carcinogen haemoglobin adduct levels in fetuses of smoking mothers were significantly higher than the levels measured in the fetuses of non-smoking mothers. A consistent observation was the apparent 2-fold reduction in the detection of fetal haemoglobin aminobiphenyl adducts when compared to matched maternal samples. The presence of a detectable adduct level in nonsmokers suggests that there may be sources of human exposure to 4-aminobiphenyl other than cigarette smoking. Since our nonsmoker population group was found not to be exposed to passive smoke, we must assume that a dietary or ambient (Hammond et al. 1993) concentration of 4-aminobiphenyl is accounting for this level of adduct in blood of these individuals.

In order to assure that the compounds detected in the GC/MS and HPLC analyses were not arising from solvent contamination, blank runs were carried out to ensure that the detected compound, 4-aminobiphenyl, was not present in any of the solvents used in extraction as well as not present as glassware contaminants. These results (HPLC and GC/MS) clearly showed that the compound isolated from maternal and fetal blood samples was 4-aminobiphenyl and that there was no contamination from extraction solvents or glassware.

The precision of the two methods (HPLC and GC/MS) to detect accurately 4-aminobiphenyl was determined by the analysis of separate aliquots of the same blood sample. This was found to be equal to 12.4% for the HPLC and 18% for the GC/MS. This latter result (GC/MS) is similar to results reported previously (Del Santo et al., 1991). Compared to results published by these authors and by Tannenbaum and colleagues (Bryant et al., 1987; Maclure et al., 1989; Bartsch et al., 1990), the sensitivity performed with the GC/MS is less than 1 pg 4-aminobiphenyl /g haemoglobin. This detection limit is achieved with HPLC as well.

As can be seen from Table 1, the levels of adducts measured by the GC/MS method are higher compared to the corresponding levels measured by HPLC. In order to quantify this difference we carried out a *t*-test; *t* was found to be equal to 6.98 and indicates a difference of 14% between the two set of results.

Using a simple extraction procedure, both GC/MS and HPLC offer a convenient methods for the detection of aminobiphenyl-haemoglobin-adducts in blood samples. However, the absolute sensitivity and the response factor being similar for both methods, the HPLC method has shorter analysis time (extraction time and effective run time) and can therefore be considered a rapid complementary method. Additional results obtained in this laboratory suggest that this method may be useful in the detection of additional aromatic amine haemoglobin adducts.

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